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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68, C07H 15/12 C12N 15/00		A1	(11) International Publication Number: WO 92/07093 (43) International Publication Date: 30 April 1992 (30.04.92)
 (21) International Application Number: PCT/US91/07685 (22) International Filing Date: 17 October 1991 (17.10.91) (30) Priority data: 598,955 17 October 1990 (17.10.90) US		 Published <i>With international search report.</i>	
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 (54) Title: IDENTIFICATION AND PATERNITY DETERMINATION BY DETECTING PRESENCE OR ABSENCE OF MULTIPLE NUCLEIC ACID SEQUENCES			
 (57) Abstract This invention relates to a process of identifying individuals or determining paternity by using Multiple Presence Polymorphic (MPP) probes. The invention also relates to Test Kits and Probes based on the process. The procedure uses multiple nucleic acid probes in separate hybridization tests, such as in different wells of a microtiter plate. The probes bind to nucleic acid sequences in some individuals, but not to others. The hybridization results convert to a unique pattern that can be used to identify an individual.			
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IDENTIFICATION AND PATERNITY
DETERMINATION BY DETECTING PRESENCE OR
ABSENCE OF MULTIPLE NUCLEIC ACID SEQUENCES

Technical Field

- 5 The present invention relates to DNA fingerprinting.

Background of the Invention

- The present invention relates to a process for producing a pattern of hybridization results that
- 10 can be used to identify and distinguish human, other animal, or plant individuals. Traditionally, this type of identification is called "Nucleic Acid (DNA) Fingerprinting" or "DNA Profiling" because the uniqueness of nucleic acid banding patterns is
- 15 conceptually similar to human fingerprints. The current method of DNA Fingerprinting requires the use of Southern Blotting. The method, according to the invention, is simple to perform, requires no interpretation, and provides results which convert
- 20 to a simple bar code for computer storage and retrieval.

The Southern Blot

- Nucleic acid hybridization has been employed as a common methodology for greater than 25 years. The
- 25 current method of DNA Fingerprinting uses a modern Molecular Biology hybridization technique known as Southern Blotting¹. The Southern blot has been a mainstay of molecular biologists since its invention in 1975. In the Southern blot method, DNA molecules
- 30 must first be isolated from other cellular components by one of a variety of extraction methods. After extraction, the somewhat pure DNA is

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then cut with one or more restriction enzymes, generating a specific set of DNA fragments.

Following DNA cutting, the fragmented DNA is separated by electrophoresis in an agarose gel.

- 5 This standard technique resolves DNA fragments based on their size (molecular weight) and has greater differentiating power than other sizing procedures. Large molecules cannot pass through the matrix under the force of an electric field as readily as small
10 molecules. Therefore, large molecules migrate more slowly. The gel resolves the DNA fragments into zones or bands of unique size.

- However, the agarose gel does not provide a stable support for the subsequent hybridization
15 reaction. Consequently, the DNA is transferred from the gel to a solid support membrane, usually composed of nylon or nitrocellulose. The DNA is transferred by either capillary action or vacuum force onto the membrane where it binds to the
20 surface. The DNA is permanently fixed to the membrane by drying or ultraviolet light cross-linking.

- The membrane is prehybridized in a special solution to block any non-specific binding sites.
25 The membrane is then placed into a solution containing labeled, denatured probe and incubated to permit hybridization to the complementary target molecules. Many factors affect the rate, extent, and specificity of the hybridization reaction.

- 30 Hybridization is the result of complementary base pairing of single stranded nucleic acids. This binding results in formation of highly specific double stranded nucleic acid. One of the single strands typically is labeled such that it can later
35 be measured or otherwise detected as part of a new hybrid. The hybrid molecules are generally

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separated from residual single stranded material, so that the hybridized label can be detected in the absence of unhybridized label. After the reaction, excess probe is washed away by a series of washing 5 steps in detergent and dilute salt solutions.

If the probe was radioactively labeled with ^{32}P , then autoradiography may be used to locate the position of any DNA band to which the probe is bound. The membrane is simply placed next to a 10 piece of X-ray film for several minutes to several days and then the film is developed. Wherever the radioactive particles strike the film, a dark region is formed and the target DNA can be identified. The probe may also be labeled, non-isotopically, and can 15 be located using the appropriate detection methodology, such as fluorescence spectrophotometry, colorimetry and chemiluminescence detection.

By Southern blotting, the size of the exact fragment which bound the labeled probe is 20 pinpointed. The Southern technique gave rise to high resolution hybridization analysis, and has formed the basis of conventional DNA Fingerprinting techniques.

Conventional DNA Fingerprinting

A conventional methodology used to show a 25 fingerprint uses probes exhibiting restriction enzyme fragment length polymorphism (RFLP)²³ or allelic variation²⁴, by Southern Blot technology. These conventional polymorphic probes have target 30 DNA fragments, which vary in length after being cut with restriction enzymes²³. These polymorphic probes are either restriction fragments or minisatellites. The present invention uses probes directed at 35 nucleic acid sequences whose presence varies in the population, referred to hereinafter as Multiple

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Presence Polymorphisms (MPP).

In 1985, Jeffries² reported that simple tandem-repetitive regions of DNA, also known as minisatellites, showed tremendous fragment length polymorphism between human individuals. Jeffries proposed that this probe, in combination with several restriction enzyme sets, could be used to produce a stable pattern of DNA bands unique to each individual tested. Furthermore, he proposed that the unique patterns could be applied to problems of human identification and determination of paternity.

The current method of DNA Fingerprinting using Southern Blotting has the following problems associated with it:

- 15 1. Time-consuming--requires one week or more to complete.
2. Difficult to perform--more than one hundred steps.
3. Requires highly skilled and knowledgeable technicians.
4. Not standardized--variable results from test to test and from lab to lab.
5. Inaccurate measurements--results require measurements of DNA fragment length, which varies with the particular electrophoresis conditions, temperature of buffer and environment, type of agarose gel used. Length measurement is

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also a highly subjective determination of the position of the DNA band.

Therefore, there is a definite need for a diagnostic or detection technique that is easy to perform, relatively fast, does not require significant expertise, and requires no subjectivity of interpretation. The present invention solves these needs by consisting of relatively few steps with only routine laboratory skills required, by being easily automatable through modification of instrumentation currently available on the market, and by providing results in about one day. Also, the results are measured in standard laboratory readers, such as an ELISA plate reader, spectrophotometer, fluorescence photometer or similar instrument. The results do not require any user interpretation; the output measurement converts to a numerical 1 (positive) or 0 (negative).

20 **Summary of Invention**

A plurality of novel Multiple Presence Polymorphism (MPP) probes is used in an ordered series of nucleic acid hybridization tests. The combined positive and negative results reveal a pattern. This pattern is individual specific and, therefore, is a Personal Identification Pattern (PIP). Specific nucleic acids used as MPP probes are directed against genetic polymorphisms that exhibit limited presence (or absence) in the population under examination.

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Description of Figures

Figure 1: Probability of Individual Sequence
Repetition During the Test

5 Figure 2: Effect of Individual Presence
Polymorphism on Overall Pattern
Repetition Frequency

Figure 3: Conventional Microtiter Plate
Configuration

Specific Description of the Invention

10 DNA Profiling by Multiple Presence Polymorphisms

The present invention avoids the use of the time-consuming and difficult to standardize and control Southern blot. The method may replace the current method of DNA Fingerprinting with one that
15 is simple and rapid to perform, requires no interpretation, and produces results that are easy to computerize.

A new method, according to the invention, tests by nucleic acid hybridization for multiple,
20 different nucleic acid sequences, which are polymorphic in terms of their presence in the general population, rather than in terms of fragment size. By analysis of MPP, nucleic acid sequences whose presence in a population of organisms is
25 exhibited less than 100% of the time, identity or paternity can be determined.

The test system exceeds the pattern uniqueness associated with current Southern blotting methods and is standardized for paternity or forensic
30 purposes. Furthermore, the PIP system is far superior to complex non-standardized Southern

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blotting, in part because it consists of simple positive or negative results. Also, background is not significant with specimens of sufficient quantity.

5 This invention includes a method for identifying an individual by multiple nucleic acid hybridization tests, without the use of gel electrophoresis and the Southern blot.

The method comprises

10 a. determining the presence of a plurality of nucleotide sequences (whole genes, fragments of genes, polynucleotides or oligonucleotides) within an individual, using a plurality of probes for MPPS in individual tests, each directed toward a unique nucleotide sequence, and

15 b. including hybridizing (binding) the probes to disrupted or extracted specimens from the individual, followed by separating hybridized probe and target from unbound probe (or the unhybridized label is otherwise eliminated), and

20 c. detecting the hybrids by any suitable measuring system, where

25 d. positive signal indicates the presence of a particular nucleic acid sequence in the individual; and the positive and negative hybridization results, taken as a whole and ordered string, provide a unique pattern ("fingerprint") for that individual, called a Personal Identification Pattern (PIP).

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After testing for the presence of each gene in a specific configuration, such as in a microtiter plate, the outcome reveals a pattern of positive or negative results. These results may be computerized
5 as 1 (positive) or 0 (negative) and converted to a simple bar code. Thus, a pattern, easy to analyze and compare, is obtained.

The preferred nucleic acid sequences are those that exhibit polymorphism with regard to presence or
10 absence in the general population (the population can be human, equine, murine, plant or any other organism), where the frequency of appearance of each "presence" polymorphism in the population affects the overall frequency of pattern recognition with
15 the optimum positive sequence frequency being 50%. This is further explained in the description of MPP probes below and in Figures 1 and 2.

The sensitivity of detection from a typical blood specimen is sufficient to allow detection
20 without target or signal amplification. This is ideal for routine paternity testing or establishing an individual's Personal Identification Pattern (PIP). But for forensic applications with limited specimen, the regions around the probes may be
25 amplified by one of a variety of target amplification methods. Alternatively, the signal may be amplified to increase detection to a measurable level.

Multiple Presence Polymorphic Probes

30 The specific nucleic acid fragments used as probes are directed against genetic polymorphisms that exhibit limited "presence" (or absence) in the population under examination.

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These are called "Presence" Polymorphisms, and the use of several of these to develop a PIP is referred to as Multiple Presence Polymorphisms (MPPs). These nucleic acid probes may be any length, such as whole genes, gene fragments, or short synthesized DNAs. In the preferred embodiment of the invention, these nucleic acid probes consist of synthesized DNA oligonucleotides and poly-nucleotides from about 10 to about 30 nucleotides long. One skilled in the art will recognize that probes of other size and/or type may also be employed.

The preferred class of candidate genes comes from RFLPs. By definition, RFLPs contain a restriction site that is polymorphic. By isolating and sequencing the region around the polymorphic enzyme site, probes are rapidly obtained that carry alterations in at least one base pair. Over 850 of these RFLPs have already been identified and most of these have had preliminary data showing the frequency of variation in the population⁹. Many hundreds of these exhibit RFLP frequencies that are useful for MPPs. Of these RFLPs, those that exhibit single bands on Southern blots are preferred MPP probes, because it is easier to determine hybridization conditions that differentiate between 100% homology and 95% or less homology.

Another group of genes from which MPP probes may be isolated are genes that exhibit a high degree of variation in the human population due to dominant and recessive traits, such as genes for brown eyes. Individuals with blue eyes completely lack the genes for brown eyes. Genetically dominant genes, like brown eyes, are good candidates for MPP probes. The reason is that in individuals lacking the particular phenotypic trait, these genes usually are completely

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absent. Many dominant genes of this type have been identified in the human population. Likewise, many genes on the X chromosome exhibit their phenotypes only in certain people; and, therefore, these genes
5 are absent in all male and some female individuals lacking the trait.

It is important to avoid probes which detect acquired disease mutations such as has been done for the oncogene ras^{7,8}. These probes may detect changes
10 as these mutations occur upon development of the disease, which would result in an individual's PIP changing. On the other hand, genes that detect inherited diseases, which are based on mutations present at birth, are potential MPP probes.
15 However, the "presence" frequency of a particular inherited disease may be too low to be useful as MPPs.

Determining the Probability of Individual Sequence Repetition in a Population

20 The frequency of "Presence" Polymorphism associated with the specific MPP probes may be determined for the population of organisms being evaluated by nucleic acid hybridization. The hybridization format used is not critical to the
25 frequency of appearance in the population. Any hybridization technology, such as liquid, dot, slot, filter, capture or sandwich assay may be used to provide accurate results. The process may be performed using Southern or Northern blots, but
30 these are not preferred. On a micro scale the process, according to the invention, is performed as an in situ hybridization test on whole cells.

Probes that detect "presence" polymorphisms are critical to MPP Analysis. Many nucleic acid

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sequences have potential to detect these sites. An MPP probe has a positive hybridization result in less than 100% of the individuals of a population. In the preferred embodiment of the invention, the 5 MPP probes have a "presence" frequency in the population of 20% to 80% with the optimum probe being present in 50% of individuals. This is explained below and in Figures 1 and 2.

The "presence" frequency of an individual MPP 10 probe is determined empirically by testing it in a statistically significant number of different individuals (for example 500 to 1,000). Assume that an MPP probe appeared 700 times out of 1,000 random individuals tested. The Empirical Presence 15 Frequency (f) is 0.7 (70%). This value is used to determine the probability that a match would occur between any two individuals in a comparison of PIPs. The probability is calculated according to Equation 1.

20 Equation 1:

Determine the probability (X_n) of each probe 25 result being repeated during the PIP test. The probability of a particular sequence being repeated during the test is defined by Equation 1, where f is the empirically determined frequency of the particular sequence in the general population.

$$(f)(f) + (1 - f)(1 - f) = X_1$$

where X_n is the Probability of an Individual 30 Sequence Repeat in the general population for this particular sequence.

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Example: where $f = 0.7$

$$X_1 = (0.7)(0.7) + (0.3)(0.3) = 0.58$$

Figure 1 shows the results of calculating these probabilities over the range of Empirical Presence

- 5 Frequencies from 0 to 1. As is obvious, if (f) is 0 or 1, the probability of a repeat is 100%. As can be seen, the curve is symmetrical with a minimum at a frequency of 0.5. Thus, the Probability of an Individual Sequence Repeat is at a minimum when the
- 10 sequence appears in the general population 50% of the time.

The Probability of an Individual Sequence Repeat is then used to determine the overall Probability of a Pattern Repeat in the population.

- 15 This probability is calculated according to Equation 2.

Determining the Probability of Pattern Repetition in a Population

- It is most preferred that all PIPs be
- 20 statistically unique in MPP analysis. Otherwise, they would not be adequate "fingerprints". The Probability of Pattern Repetition in a population is a function of (1) the number of probes tested, in combination with (2) the Probability of Individual
- 25 Sequence Repetition of each MPP probe used.

- The Probability of Pattern Repetition can be made far less than the entire population of the earth (about 5 billion people) by using a sufficient number of probes. Therefore, the Probability of
- 30 Pattern Repetition must be less than 1 in 10^{10} to be functionally unique. In accordance with the invention, any number of probes may be used. For

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practicality sake, a sufficient number of probes is used to achieve a statistically significant level of uniqueness. As used herein, a statistically significant number can be any number depending on 5 the user's intent, but is preferably greater than about 20 probes. Although the upper limit of probes used need not be defined, 90 probes is the current commercial limit because the preferred embodiment of MPP Analysis is in a 96-well microtiter plate.

10 Thus, allowing 6 wells for controls, the test preferably uses up to 90 wells for MPP Analysis. The preferred embodiment comprises from 20 to 90 probes. For MPP analysis with 90 probes, the Probability of Pattern Repetition can be less than 1 15 in 10^{27} , as shown in Figure 2. This far exceeds any known application.

Figure 2 shows the results of calculating the Probability of pattern Repetition using Equation 2. As can be seen, this curve also has its limit at a 20 "presence" frequency of 50% (0.5). At this presence frequency, the Probability of Pattern Repetition with 90 probes at 50% presence frequency is less than 10^{-27} .

Equation 2:

25 Determine the probability of a particular set of probe tests being repeated in the population. The probability of an ordered set of results (a pattern) being repeated in the general population is the multiple of all 30 individual sequence repeat probabilities. This is formulated by Equation 2, where X_1, X_2, X_3 through X_n are individual sequence repeat probabilities calculated from Equation 1.

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$$(X_1) (X_2) (X_3) \dots (X_n) = P$$

where P is the Probability of Pattern Repetition in the general population.

Example: Where all X's = 0.5 and 90
5 sequences are analyzed. When all X's = 0.5,
the probability of a pattern repetition is at its minimum.

$$(0.5_1) (0.5_2) (0.5_3) \dots (0.5_{90}) = (0.5)^{90}$$
$$= 8 \times 10^{-28}$$

10 Kits

The Kits referred to in this invention can be used to determine both identity and paternity. Kits may contain reagents, filters, plates, and all the necessary probes to perform an analysis. All the
15 probes are used simultaneously; but in distinct tests, such as in a microtiter plate, to produce a unique pattern of positive and negative results, which is called a personal Identification Pattern (PIP).

20 One skilled in the art will recognize that the invention may comprise a kit. Kits are for detection of "presence" polymorphisms and may contain Multiple Presence Polymorphism probes, filters or plates, and reagents for processing in
25 accordance with the invention. The test system, comprises a set of nucleic acid probes (the greater the number of probes used the less the likelihood of pattern repetition), such probes being used in a manner that permits hybridizing separately to
30 portions of the test person's nucleic acids to provide separate results for each probe test, so

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that by combining the test results in a predetermined, ordered sequence, such as in a microtiter plate format, the results form a pattern that is unique for the individual being tested, a
5 PIP. The reagents include a) materials for processing, such as a) filters or plates, b) solutions for hybridizing, washing, and detection, and c) specific nucleic acid probes for MPPs, and d) DNAs and probes for positive and negative controls.

10 Labels

Many different types of labels are used effectively with MPP probes. A good description of the many labels and their accompanying labeling methodology is contained in DNA Probes by Keller and
15 Manak⁵. Examples of various labels include biotin or any of its derivatives, fluorophores, chemiluminescent or bioluminescent tags, digoxigenin, enzymes, mercury, haptens, bromine, DNA binding proteins, Europium-psoralen, lanthanides,
20 modified aminos, modified nucleotides, sulfonates, AAIF, DNP, radioisotopes, or other molecules that can be subsequently detected⁵. Probes are labeled by techniques including, but not limited to nick-translation, photoaffinity labeling, random priming,
25 direct enzyme linking, end labeling, chemical modification, tailing, transcription, direct synthesis, or conjugation⁵.

Cell Lysis/Nucleic Acid Extraction

Because determination of MPPs does not require
30 restriction enzyme digestion, the specimens do not need to be highly purified. This permits simple cell and organism disruption without nucleic acid

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extraction. Cells are lysed by using guanidinium thiocyanate, SDS, or other surfactants.

Nucleic Acid Capture

The crude lysates prepared as described above
5 are used directly in the hybridization reaction with
capture or sandwich assays or filtered to capture
the sample nucleic acids for dot, spot, or
microtiter blots. For some assays it is possible to
spot directly the cells and disrupt them on the
10 filter (filter in situ).

Blotting may be by Dot Blot or Slot Blot
Manifolds, simple spot blots without the manifold,
direct "filter in situ" blotting of cells, or other
methods. These procedures are described in a
15 variety of Lab Manuals and text books^{1,10,11}.

Hybridization Reaction

Different size probes exhibit different binding
affinities. In addition, the percent homology
between the probe and target affects the binding
20 affinity. The hybridization and washing conditions
chosen for MPP are able to differentiate between
presence or absence of a specific nucleotide
sequence or portion of that sequence used for the
probe. Establishing these conditions sets the
25 stringency. Appropriate hybridization conditions
are estimated using the formulas described in
Hybridization Formulas below, but final optimum
conditions are empirically determined with each
individual probe.

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Detection/Measurement

After hybridization, the detection system used may be radioisotopic or non-isotopic such as colorimetric, fluorometric, or luminescent. The
5 only criterion is easy detection above background. Detection methodology depends on the nature of the label used to modify the probe.

Hybridization Formulas

Equation 3: Determining the melting point
10 temperature (T_m) for DNA:DNA Hybridization using Long Probes. T_m is the point at which the DNA:DNA hybrids begin to separate (denature) into single strands.

$$T_m = 81.5^{\circ}\text{C} + 16.6 \log M + 0.41 (\%G+C) - 0.7 (\%\text{Formamide})$$

where $M = [\text{Na}^+]$ in moles/liter

Example: for a probe of 250 bases containing 45% G+C, in 1M Na^+ and 50% formamide:

$$T_m = 81.5 + 16.6 \log (1) + 0.41 (45) - 0.7 (50)$$

20 $T_m = 81.5 + 0 + 18.5 - 35 = 65^{\circ}\text{C}$

Equation 4: Determining the optimum hybridization temperature (T_{byb}) for DNA:DNA Hybridization using Long Probes. This is the temperature at which the hybridization reaction should be performed.

25 $T_{byb} = T_m - 20^{\circ}\text{C}$

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Using the example from above gives:

$$T_{hyb} = 65^\circ\text{C} - 20^\circ\text{C} = 45^\circ\text{C}$$

Equation 5: Determining the T_m for short oligonucleotide probes of 14 to 40 bases. The T_m

5 formula for short probes has been empirically determined; it is a function of the relative base pair content of the probe and target. Standard sodium ion concentration without formamide are the reaction conditions used.

10 $T_m = 4^\circ\text{C per GC pair} + 2^\circ\text{C per AT pair}$

Example: using a 20-mer with a GC content of 50%, the T_m would be determined as follows:

$$T_m = 4^\circ\text{C}(10) + 2^\circ\text{C}(10) = 60^\circ\text{C}$$

Equation 6: Determining the T_{hyb} for short oligonucleotide probes.

15

$$T_{hyb} = T_m - 5^\circ\text{C}$$

Therefore, T_{hyb} for the above 20-mer would be 55°C.

Example 1.

20 The following is an example using DNA hybridization in a Microtiter Dot Blot format. Many of the specific steps referred to in Example 1 are found in many laboratory manuals such as Maniatis et al.¹⁰

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Specimen for Human DNA Profiling

Blood, semen, tissue, or any specimen containing nucleic acids will be used. Cells will be disrupted to liberate the nucleic acids in a 5 medium that prevents their breakdown. The nucleic acids may or may not be extracted, depending on the requirements of the specimen or organism.

Nucleic Acid Extraction: From whole blood

1. Collect blood specimen in EDTA anticoagulant.
- 10 2. Centrifuge blood at 400 x g for 5 minutes at room temperature to separate the red cells from the white cells. The white cells will form a layer on top of the red cells. Carefully remove the white cell layer with a Pasteur pipette and place into a new polypropylene test tube.
- 15 3. Resuspend cells and add Lysis Buffer (20 mM Tris-HCl, pH 7.5; 20 mM NaCl; 20 mM EDTA) to bring the total volume to 2 ml. Lyse cells by adding 0.1 ml of 10% SDS.
- 20 4. Add 0.2 ml of 20mg/ml Proteinase K. Incubate at 50°C for 1 hour to digest proteins.
- 25 5. Extract nucleic acids by adding an equal volume (2.3 ml) of PCI (phenol:chloroform:isoamyl alcohol; 25:24:1) and vortexing vigorously for 1 minute. Centrifuge for 10 minutes to separate the two phases.

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6. Transfer the upper, aqueous layer to a new tube. Extract again with an equal volume of PCI.
7. Again transfer the upper, aqueous layer to a new tube. Add an equal volume of chloroform:isoamyl alcohol (24:1) and vortex for 1 minute. Centrifuge for 10 minutes.
5
8. Transfer the aqueous layer to a new tube. Add 1/10 volume (0.2 ml) of 2 M Na Acetate, pH 5.0 and 2 volumes of ice cold 100% ethanol.
10 Incubate at -20°C for at least 1 hour. Centrifuge for 10 minutes in a microfuge.
9. Carefully remove the ethanol from the tube leaving the DNA pellet. Wash the pellet by adding 2 ml of cold 70% ethanol and gently vortexing. Centrifuge for 10 minutes in a microfuge.
15
10. Carefully remove the ethanol and drain the excess ethanol by inverting the tube onto a paper towel or by drying under vacuum. Do not allow the pellet to become completely dry or it will be difficult to redissolve.
20
11. Dissolve the DNA/RNA in 0.5 ml of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.

OPTIONAL REMOVAL OF RNA STEPS 12-16

- 25 Depending on the application, RNA may have positive or negative benefits. Therefore, this procedure will be performed to remove the RNA when necessary. Removal of RNA may be necessary if the

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MPP probes cross react to ribosomal RNA or some other RNA resulting in background.

12. Add RNase A to a final concentration of 100 ug/ml and incubate at 37°C for 30 to 60 minutes.
- 5
13. Add SDS to a final concentration of 0.5% and Proteinase K to a final concentration of 100 ug/ml. Incubate at 37°C for 30 to 60 minutes.
- 10
14. Extract the solution with PCI as in steps 4 and 5.
15. Extract the solution with Chloroform as in step 7.
16. Precipitate, wash, and redissolve the DNA as in steps 8-11.
- 15
17. Store the DNA solution at -20°C.

Blotting the Nucleic Acids on a Membrane

- The individual's DNA sample will be split into multiple different parts (up to 90 depending on the number of individual probe tests to be performed).
- 20 The specimens will be blotted onto a membrane filter to prepare for hybridization with multiple different probes.

The preferred method to perform the blotting is in a Microtiter Dot Blot format using a Millipore 25 microtiter plate with a nylon membrane attached to the bottom of the wells (or the equivalent from other manufacturers). This type of plate will permit filtering, hybridizing with different probes

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in each well, and washing of samples without interference (cross contamination or bleeding) from other wells.

18. Assemble a Vacuum Plenum apparatus suitable for
5 use with the microtiter filter plate (any of the commercially available 96-well Vacuum Plenums will suffice).
19. Place the microtiter filter (nylon) plate into the plenum. Wet the membrane with 0.5 M NaOH
10 by adding 0.2 ml to each well.
20. Remove a sample of the individual's nucleic acid solution and denature by adding 10 M NaOH to a final concentration of 0.5 M. If RNA is in the sample, continue to step 21 without
15 delay or the RNA will be destroyed.
21. Add 200 ul of the denatured nucleic acid solution to each well being tested.
22. Apply a vacuum to the manifold to suck the contents of the wells through the porous
20 membrane. The nucleic acids will bind to the membrane.

OPTIONAL STEP FOR USE WITH RNA CONTAINING SAMPLES

STEP 23

23. Add 200 ul of 1 M Tris-HCl, pH 7.0 and filter
25 as in step 22.
24. After the samples have been completely removed from the wells, disassemble the apparatus and

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bake the membrane at 60°C for 30 minutes or air dry completely.

Labeled Probes

The Probes will be composed of either DNA or RNA. The probe labeling methodology used will depend on the type of label employed. The only requirement for the label is definitive detectability above background. The preferred label will yield a color change on the filter that can be easily measured in a standard ELISA reader. For example, biotinylated probes will be suitable for this type of detection system. For labeling oligonucleotides, chemical attachment⁵ or photo-biotinylation⁶ yields the best probe.

15 Hybridization Reaction

The oligonucleotide probe, containing a label that will be subsequently detected, will be added to the medium containing the target (individual's) nucleic acids. The solution will be incubated at appropriate hybridization conditions, including salt, formamide, temperature and time to allow the probe and target to combine (hybridize).

20 The preferred Prehybridization Solution for working with nylon membranes will contain the following chemicals:

	0.1 mg/ml	Yeast tRNA (sonicated herring sperm DNA may be substituted if filtration to remove reagents is not used for subsequent steps)
30	0.2%	Ficoll
	0.2%	Bovine Serum Albumin (or Glycine)

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0.2%	Polyvinyl Pyrrolidone
6X	SSC (sodium chloride, sodium citrate)
1%	SDS (sodium dodecyl sulfate)

- 5 25. Prehybridize all wells of the membrane with the appropriate Prehybridization Solution to block non-specific binding of the probes. In the preferred procedure, the Prehybridization Solution is the same as in the hybridization
10 for each probe. Prehybridize at 37°C for 30 to 60 minutes.
26. Following prehybridization, remove the excess solution by filtering under vacuum or by pouring out the solution.
- 15 27. Rinse the wells one time with 6X SSC, and filter to remove excess reagent.

In the Microtiter Dot Blot format, it is preferred that the temperature used for hybridization and washing be the same for all wells.

- 20 This will make processing much easier. The stringency of the reaction, which will set the desired probe specificity (or percent homology), will be controlled by the salt or formamide concentration. (See Hybridization Formulas above
25 for detailed equations showing how to determine the appropriate hybridization conditions.)

The hybridization conditions will be able to differentiate between oligonucleotides of 20 base pairs with 100% homology to the target nucleic acid
30 sequence from targets exhibiting 95% homology or less. The approximate reaction conditions for this

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degree of distinction with 20-mers is shown in the following example.

The preferred hybridization Solution for working with nylon membranes will contain the

5 following chemicals:

	0.1 mg/ml	Yeast tRNA (Sonicated Herring Sperm DNA may be substituted if filtration to remove reagents is not used in subsequent steps)
10	0.2%	Ficoll
	0.2%	Bovine Serum Albumin (or Glycine)
	0.2%	Polyvinyl Pyrrolidone
	6X	SSPE
	1%	SDS (sodium dodecyl sulfate)
15	Optional	Formamide (50%)
	Optional	Dextran Sulfate (5-10%)

There are many variations of this basic reagent. The final concentration of these reagents will depend upon the membrane material (nylon,
20 nitrocellulose, or paper), the length and GC content of the probes, and the type of label used (eg. radioisotope, biotin, luminescent, etc.).

28. Mix each labeled probe (30 to 90 different solutions will be prepared) into the
- 25 Hybridization Solution to the appropriate final concentration (the final concentration depends on the nature of the label). For biotinylated probes use 0.1 to 0.2 mg/ml in the hybridization Solution.
- 30 29. Place 0.2 ml of Probe Solution 1 into Well 1, 0.2 ml of Probe Solution 2 into Well 2, and

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continue until all Probe Solutions have been added.

30. Incubate at the appropriate temperature for the specific probe length and hybridization
5 Solution used. For a biotinylated 20-mer with a 50% GC content in the Hybridization Solution described above without formamide or dextran sulfate, the T_{hyb} will be about 55°C (see Hybridization Formulas above for calculations).

- 10 31. Hybridize for 16 hours at 55°C.

Washing

Excess, unbound probe will be eliminated by physical separation or by another means, such as chemical degradation, to destroy unbound probe.

- 15 32. Remaining probe, which will be hybridized to its specific, complementary sequence, will be detected.

33. Rinse each well three times with Wash Solution
20 (0.2 ml of 0.1X SSC, 1% SDS) at room temperature to remove unbound, excess probe. The Wash Solution concentrations may be varied to accommodate different probes at the same washing temperature.

- 25 34. For the final high stringency wash, add 0.2 ml of Wash Solution to each well and incubate at 55°C for 30 minutes to remove mismatched probe. Filter or pour off the excess wash solution. Repeat this wash one time.

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Detection

Detection methodology will depend on the nature of the label used to modify the probe. A typical method for developing biotinylated probe signal is 5 described below. It is important not to let the membrane completely dry between anyof these steps. All incubations will be at room temperature.

35. To each well add 0.2 ml of Blocking Solution 1 (3% BSA in 50 mM Tris-HCl, pH 7.4, 20 mM NaCl, 10 0.3% Tween-20, 0.01% Thimerosal) and incubate for 15 minutes.
36. Filter or pour off the excess Blocking Solution. To each well add 0.2 ml of Enzyme Conjugate (1 ug/ml alkaline phosphatase-streptavidin conjugate in Blocking 15 Solution) and incubate for 10 minutes.
37. Filter or pour off Enzyme Conjugate Solution. Wash each well 3 times in 0.2 ml of Washing Solution 2 (50mM Tris-HCl, pH 7.4, 20mM NaCl, 20 0.3% Tween-20, 0.01% Thimerosal) for 5 minutes each. Filter or pour off Washing Solution 2 between each wash.
38. Add 0.2 ml of Substrate Solution (NBT/BCIP) to each well and incubate for 15 minutes to 2 25 hours in the dark until color develops.
39. Stop development by washing each well in deionized water. Dry the wells on filter paper.

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Example 2.

This technique will substitute tetramethylammonium chloride (or tetraethylammonium chloride) for sodium chloride in the hybridization 5 Solution⁶. All other steps will be the same as in Example 1. This salt change avoids the effects of relative %GC content on the stability of hybrids formed. This will be useful for preparing MPP probes because the oligonucleotide sequences do not 10 need similar %GC content to perform similarly in the hybridization reaction. The reagent system will be much simpler. This alternative method has been previously applied to the analysis of ras gene mutations in human colorectal cancers^{7,8}.

15 **Hybridization Reaction**

As in Example 1, the biotinylated oligonucleotide probe, will be added to the medium containing the target (individual's) nucleic acids. The solution will be incubated at appropriate 20 hybridization conditions, including the Hybridization Solution described below. The temperature and time to allow the probe and target to combine (hybridize) are discussed below.

The preferred Prehybridization Solution for 25 working with nylon membranes will contain the following chemicals:

30	0.1 mg/ml	Yeast tRNA (sonicated herring sperm DNA may be substituted if filtration to remove reagents is not used for subsequent steps)
	0.2%	Ficoll
	0.2%	Bovine Serum Albumin (or Glycine)
	0.2%	Polyvinyl Pyrrolidone

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3M	Tetramethylammonium Chloride
50 mM	Tris-HCl, pH 8.0
2 mM	EDTA
1%	SDS (sodium dodecyl sulfate)

- 5 25. Prehybridize all wells of the membrane with the appropriate Prehybridization Solution to block non-specific binding of the probes. In the preferred procedure, the Prehybridization Solution is the same as is used during hybridization for each probe. Prehybridize at 56°C for 30 to 60 minutes.
- 10 26. Following prehybridization, remove the excess solution by filtering under vacuum or by pouring out the solution.
- 15 The hybridization conditions have been determined to differentiate between oligonucleotides of 20 base pairs with 100% homology to the target nucleic acid sequence from targets exhibiting a single base pair mismatch. Although the exact reaction conditions will be determined empirically, the approximate reaction conditions for this degree of distinction with 20-mers is shown in the following example.
- 20 27. Mix each labeled probe (30 to 90 different solutions will be prepared) into the Hybridization Solution to the appropriate final concentration (the final concentration depends on the nature of the label). For biotinylated probes use 0.1 to 0.2 mg/ml in the Hybridization Solution.
- 25
- 30

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28. Place 0.2 ml of Probe Solution 1 into Well 1, 0.2 ml of Probe Solution 2 into Well 2, and continue until all Probe Solutions have been added.
 - 5 29. Incubate at the appropriate temperature for the specific probe length and hybridization Solution used. The T_{hyb} will be about 56°C (see Hybridization Formulas above for calculations).
 30. Hybridize for 1 to 16 hours at 50C.
- 10 Washing
- Excess, unbound probe will be eliminated by physical separation or by another means, such as chemical degradation, to destroy unbound probe. Remaining probe, which will have hybridized to its specific, complementary sequence, will be detected.
- 15
 31. Remove the excess Hybridization Solution by pouring out the solution.
 32. Rinse each well three times with Wash Solution 1 (0.2 ml of 2X SSPE, 1% SDS) for about 5 minutes at room temperature to remove unbound, excess probe. The Wash Solution concentrations may be varied to accommodate different probes at the same washing temperature.
 - 20
 33. For the final high stringency wash, add 0.2 ml of High Salt Wash Solution (5X SSPE, 1.0% SDS) to each well and incubate at 59°C to 73°C (depending on the probe) for 5 minutes to remove mismatched probe. Filter or pour off the excess wash solution.
 - 25

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34. Wash filters twice in Hybridization Solution without 0.2% Ficoll, 0.2% Bovine Serum Albumin (or Glycine), 0.2% Polyvinyl Pyrrolidone, or Yeast tRNA(or DNA) for 1 hour at 59°C to 60°C.

5 Results of Examples 1 or 2

- Positive results will be indicated by a signal that can be discriminated above background (negative control). With biotinylated probes and alkaline phosphatase conjugates, the signal will be
10 purplish-blue.
- Positive results will be scored as a 1, and negative results will be scored as a 0. Results will be entered into a computer program according to a previously determined configuration for comparison
15 to other patterns. A typical microtiter plate pattern is shown in Figure 3.

Analysis of Results

a. For Individual Identification

- A single individual will exhibit a stable
20 pattern of positive and negative results with a specific probe set (for practical reasons in a microtiter plate, this will be up to 90 MPP probes). This individual will give the same pattern in any analysis. Therefore, comparison to previous results
25 will always reveal the identical pattern for the same set of gene fragments. All common specimens taken from separate tissues of the same individual will exhibit the same pattern. The exception will be identical twins. No two non-identical
30 individuals will have the same pattern if sufficient number of MPP probes is used to achieve a level of

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uniqueness of less than 1 in 10^{10} . Using MPP probes with a presence frequency of 0.5, only 33 probes are required, according to Equation 2. Using probes with a "presence" frequency of 0.2, 60 probes are 5 required for a 1 in 10^{10} level of uniqueness. Thus, the "presence" frequency combined with the number probes used determine the PIP uniqueness.

Probes to the HLA DQ-alpha locus have been used to assist in ruling out murder suspects. In some 10 cases, specimens such as hair from the victim and from the crime scene can be tested to see if they exhibit different HLA DQ-alpha alleles. There are 6 distinguishable forms of this locus. Comparisons are made of the HLA DQ-alpha genetic makeup of the 15 suspect, victim, and assailant. This information can be used to rule out some suspects, but cannot be used to identify a suspect with much accuracy. The range of probabilities for identity due to chance using information from the HLA DQ-alpha locus is 20 between 1 in 10 and 1 in 100. MPP Analysis can have better accuracy by up to 25 orders of magnitude, and is likely to be used to rule in potential suspects!

Also, care is taken to avoid MPP probes that detect mutagenic diseases, such as in cancerous 25 cells that may alter certain of their genes during the individual's lifetime. This alteration would result in a change to the individual's PIP.

For PIPs, the number of MPP probes used is a function of the Probability of Individual Sequence 30 Repetition of each probe, and the Probability of Pattern Repetition needed by the user, as described above in Specific Description of the Invention. For practical reasons using a microtiter plate format, the number of MPP probes used is 90 or less. One 35 important use of PIPs is to identify persons from blood, sperm or other tissue samples by matching

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unknowns with specimens taken from the suspect individual.

b. For Paternity

Similar to PIPs, the number of MPP probes used
5 for paternity determination is a function of the Probability of Individual Sequence Repetition of each probe, and the Probability of Pattern Repetition needed to establish the relationship.

Comparison of patterns between the mother and
10 child will reveal differences in results of specific genes. Comparison of these different genes with the presumptive father will show whether these genes came from the father. A potential match will be achieved when the father contributes all the genes
15 that are different from the mother.

In some cases the number of child's genes which differ from the mother may be only a few. This has the effect of increasing the number of potential fathers who could have a perfect match. Therefore,
20 it is desirable to determine the probability that a match is unique. The probability that the putative father is the real father is determined from a combination of frequencies of the child's genes that differ from the mother.

25 If the number of gene variations from the mother is sufficient, then a probability will be determined that the presumptive father is indeed the father. The required exclusion frequency is probably no more than 1 in 10^{10} . Using MPPs with a
30 Presence Frequency of 50%, this exclusion frequency requires 16 probe differences between the mother and child, according to Equation 2.

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Claims

1. A process comprising
 - a) selecting a plurality of unique nucleotide sequences;
 - 5 b) hybridizing each unique sequence with a suitable probe; and
 - c) analyzing the type and number of positive signals.
- 10 2. The process of claim 1 wherein the unique nucleotide sequence comprises a sequence having a multiple presence polymorphism.
3. The process of claim 2 wherein the unique nucleotide sequence comprises nucleotide sequences which occur in a portion of the population.
- 15 4. The process of claim 1 wherein the probe contains at least 10 nucleotides.
5. The process of claim 1 wherein between about 20 and 90 nucleotide sequences are selected.
- 20 6. The process of claim 5 wherein step "c" of claim 1 provides an identifiable pattern of positive and negative signals.
7. The process of claim 3 wherein the probe sequences are present in about 10% to about 90% of the population.
- 25 8. The process of claim 7 wherein the probe sequences are present in about 50% of the population.

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9. The process of claim 2 wherein the nucleotide sequences exhibit restriction fragment length polymorphisms.
10. A process for determining paternity comprising
 - 5 a) performing the process of claim 1 for a maternal set of nucleotide sequences;
 - b) performing the process of claim 1 for an offspring's set of nucleotide sequences;
 - c) performing the process of claim 1 for a
 - 10 putative paternal set of nucleotide sequences;
 - d) analyzing the results of steps "a" and "b" for similarities and differences; and
 - e) comparing the differences found in step "d" with the results of step "c".
- 15 11. A kit comprising:
 - a plurality of nucleotide sequences, each having a multiple presence polymorphism; and
 - a suitable container.
12. The kit of claim 11 further comprising at least
 - 20 one of the following:
 - a filter;
 - a microtiter plate;
 - a hybridizing reagent;
 - a washing reagent;
 - 25 a detection or labeling reagent; and
 - nucleotide sequences for positive and negative controls.

1 / 3

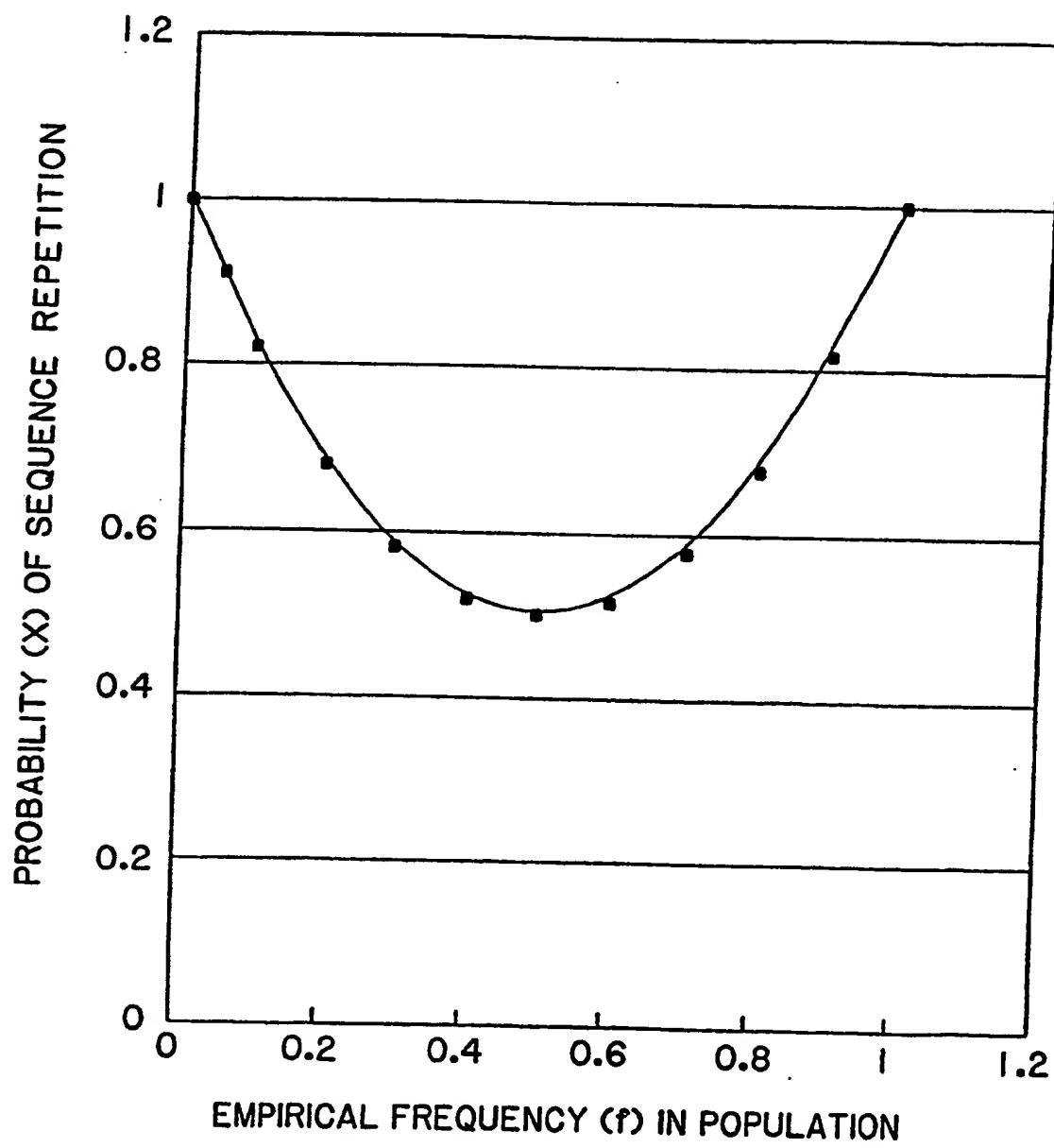


FIG. 1

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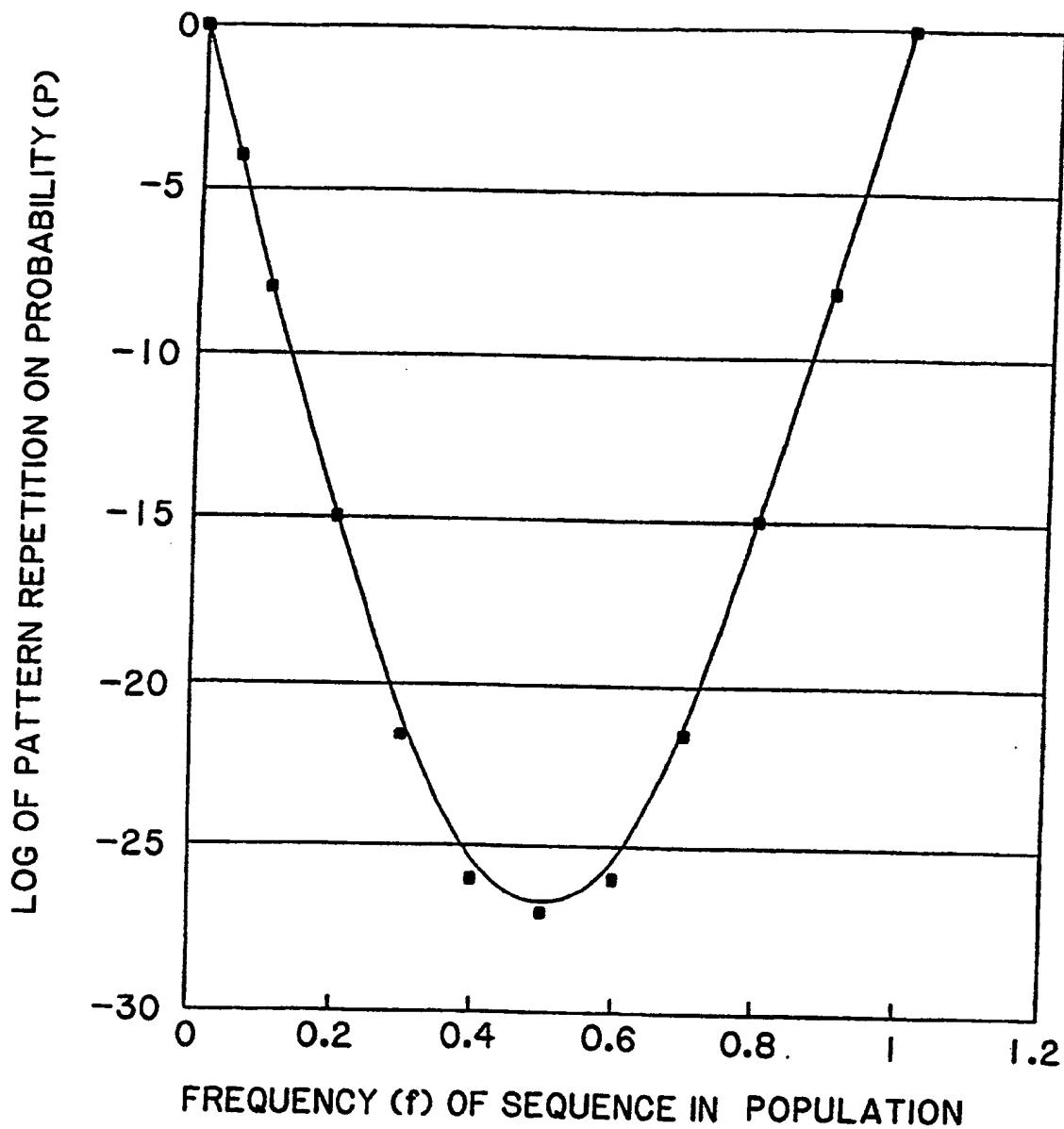
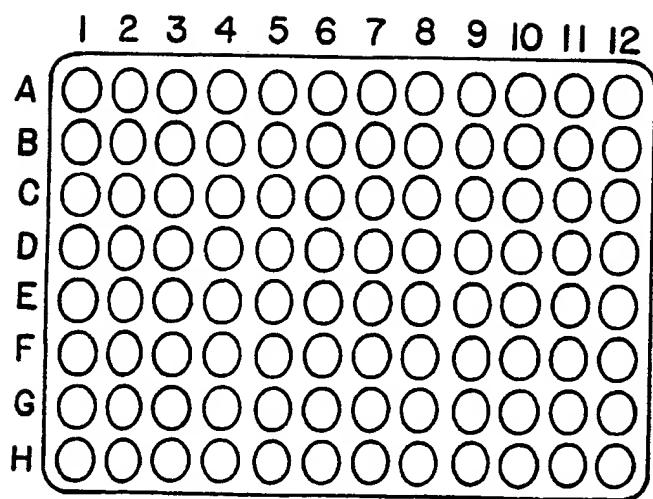


FIG. 2

SUBSTITUTE SHEET

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**FIG. 3**

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07685

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC
 I.P.C.(5): C12Q 1/68; C07H 15/12; C12N 15/00
 U.S. Cl.: 435/6; 536/27; 935/77,78

II. FIELDS SEARCHED

Minimum Documentation Searched ?

Classification System	Classification Symbols
U.S. Cl.	435/6, 536/27; 935/77,78

Documentation Searched other than Minimum Documentation
 to the Extent that such Documents are Included in the Fields Searched *

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages †‡	Relevant to Claim No. †§
X	Bio/Technology. Volume 6. issued September 1988. Beckmann. "Oligonucleotide Polymorphisms: a New Tool for Genomic Genetics". pages 1061-1064. see entire document.	1-12

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

06 December 1991

Date of Mailing of this International Search Report

14 FEB 1992

International Searching Authority

ISA/US

Signature of Authorized Officer

Jerome Massie
Jerome Massie

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